MOLECULAR AND CELLULAR BIOLOGY, Mar. 1991, p. 1344–1352
0270-7306/91/031344-09$02.00/0

Vol. 11, No. 3

Mutant p53 Tumor Suppressor Alleles Release *ras*-Induced Cell Cycle Growth Arrest

GEOFFREY G. HICKS, SEAN E. EGAN,† ARNOLD H. GREENBERG, AND MICHAEL MOWAT*

Manitoba Institute of Cell Biology and the University of Manitoba, 100 Olivia Street, Winnipeg, Manitoba, Canada R3E 0V9

Received 13 September 1990/Accepted 18 December 1990

Overexpression of an activated *ras* gene in the rat embryo fibroblast line REF52 results in growth arrest at either the G_1/S or G_2/M boundary of the cell cycle. Both of the DNA tumor virus proteins simian virus 40 large T antigen and adenovirus 5 E1a are able to rescue *ras* induced lethality and cooperate with *ras* to fully transform REF52 cells. In this report, we present evidence that the wild-type activity of the tumor suppressor gene p53 is involved in the negative growth regulation of this model system. p53 genes encoding either a $p53^{Val-135}$ or $p53^{Pro-193}$ mutation express a highly stable p53 protein with a conformation-dependent loss of wild-type activity and the ability to eliminate any endogenous wild-type p53 activity in a dominant negative manner. In cotransfection assays, these mutant p53 genes are able to rescue REF52 cells from *ras*-induced growth arrest, resulting in established cell lines which express elevated levels of the *ras* oncoprotein and show morphological transformation. Full transformation, as assayed by tumor formation in nude mice, is found only in the $p53^{Pro-193}$-plus-*ras* transfectants. These cells express higher levels of the *ras* protein than do the $p53^{Val-135}$-plus-*ras*-transfected cells. Transfection of REF52 cells with *ras* alone or a full-length genomic wild-type p53 plus *ras* results in growth arrest and lethality. Therefore, the selective event for p53 inactivation or loss during tumor progression may be to overcome a cell cycle restriction induced by oncogene overexpression (*ras*). These results suggest that a normal function of p53 may be to mediate negative growth regulation in response to *ras* or other proliferative inducing signals.

p53 is now considered to be a tumor suppressor gene (reviewed in reference 35). Originally, p53 appeared to be a dominant-acting nuclear oncogene by virtue of its ability to immortalize rodent fibroblasts (28, 51), complement *ras* in transformation of primary rat embryo cells (15, 28, 45), and enhance the tumorigenic phenotype of cell lines (14, 31, 60, 65). It was on the basis of these in vitro studies that p53 was grouped with simian virus 40 (SV40) large T antigen, adenovirus 5 (Ad5) E1a, and *myc* as an immortalizing oncogene. On the other hand, results from our laboratory and others indicated that p53 is inactivated in several tumor systems (6, 25, 37, 42, 52, 63, 64). Selection for the loss of p53 also appeared to be an advantage in tumor progression. These results were in conflict with the dominant nuclear oncogene model.

The discrepancy between in vitro and in vivo systems has since been resolved by the realization that the p53 alleles used in the aforementioned immortalizing and transforming assays and those reported in Friend virus (FV)-induced erythroleukemia p53-positive lines all contain point mutations. Although these mutations do not map to a hot spot, they do fall within highly conserved regions of the protein (for a review, see reference 29; 3, 43, 44) and generally result in a common conformational change in the protein identified by the loss of an epitope for monoclonal antibody PAb246 (10, 19, 40, 56, 66). Such mutations lead to overexpression of a stable mutant p53 protein with the ability to oligomerize with both itself and wild-type p53 (wtp53) proteins (12, 21, 33, 51). These data support the model that the oncogenic activity of mutant p53 proteins may be to effectively eliminate any wild-type p53 activity in the cell through a dominant negative mechanism (24). Hence, wtp53 activity is lost by an increased expression of a more stable but nonfunctional mutant p53 which may still compete for natural p53 target interactions. In addition, wtp53 is sequestered into nonfunctional oligomers with the mutant proteins. Further, it is now well established that many tumor virus proteins tightly bind the antioncogene product of p53 (reviewed in reference 36). This event is thought to functionally parallel the loss of wtp53 seen in many human tumors by mutation or deletion (3, 58).

Consistent with these results, the wtp53 gene has recently been shown to possess tumor-suppressing activity (13, 18, 26). The mechanism of p53 tumor-suppressing activity is likely to be directly related to its activity as a negative growth regulator. p53 has been implicated in cell cycle regulation. Its expression is under cell cycle control (41, 48), and recently it has been found to interact with the cell cycle regulator *cdc2* kinase (1, 7, 57). Recent evidence has also demonstrated that wtp53 has antiproliferative activity (4, 30, 38, 39).

Negative growth regulation in the rat embryo fibroblast line REF52 can be induced by the T24/EJ *ras* oncogene (20). Expression of this transfected gene to levels higher than 10 to 30% of the endogenous rat p21 level results in growth arrest and subsequent lethality of the cell (20). REF52 cells can be 'rescued' from *ras*-mediated cell cycle arrest by cotransfection of SV40 large T antigen, Ad5 E1a, and to limited extent transcriptionally activated c-*myc* (20, 27, 32). While these nuclear oncogenes are nontransforming in REF52 cells by themselves, in cotransfections with *ras* the tumor virus genes allow for a 10- to 100-fold increase in oncogenic *ras* expression and full transformation. The ability of REF52 cells to tolerate high levels of *ras* is dependent on the stable expression of the cooperating viral nuclear oncogene. In temperature-sensitive large T antigen-plus-*ras*

* Corresponding author.
† Present address: Whitehead Institute, Cambridge, MA 02142.

transfectants, switch to the nonpermissive temperature results in cell cycle growth arrest at either the G_2/M or G_1 point in the cell cycle (27). Ridley et al. have shown similar results in Schwann cells; these cells also growth arrest at G_1 and G_2/M in response to elevated levels of T24/EJ *ras* and can be rescued by the transfected activity of large T antigen (49).

At the moment, it is not clear whether the ability of these tumor virus nuclear oncogenes to induce tolerance to high levels of *ras* expression in REF52 cells is due to a block in the negative growth control pathway or to an enhancement of downstream positive signals. An example of the latter complementing activity is the ability of c-*myc* to cooperate with *ras* in REF52 cell transformation (32). This cooperation, however, was dependent on vectors that coexpress a transcriptionally enhanced c-*myc* linked to the *ras* gene and occurs with a frequency 20 to 30 times less than for E1a-plus-*ras* transfections. Therefore, it is likely not working through the same mechanisms as the tumor virus proteins. We hypothesized that the viral protein cooperation was more likely through the loss of a cellular tumor suppression mechanism. Hence, we were interested in determining whether REF52 cells could be rescued from *ras*-induced growth arrest by a dominant transforming mutant p53 allele that would eliminate the tumor-suppressing and antiproliferative activities of the endogenous wtp53 through a similar mechanism. In this report, we clearly demonstrate that the expression of a mutant p53 allele can efficiently circumvent the negative growth regulation induced by high levels of T24/EJ *ras* expression and allow transformation of REF52 cells. Further, the ability of REF52 cells to tolerate high levels of *ras* expression cannot be facilitated by the expression of the endogenous p53 or by an introduced wtp53 allele.

MATERIALS AND METHODS

Cells and transfection. Low-passage REF52 cells (20) were maintained in Dulbecco's alpha minimum essential media supplemented with 10% fetal bovine serum. REF52 cells were seeded at a density of 10^6 cells per 100-mm dish 24 h prior to transfection. Plasmids were cotransfected by the calcium phosphate method (Cellfect; Pharmacia); precipitates were removed 8 to 10 h posttransfection and then glycerol shocked for 2 min. At 24 h posttransfection, cells were transferred from the original 100-mm plate to eight 150-mm plates and drug selected in either hygromycin B (100 μg/ml; Sigma) or G418 (400 μg/ml; Geneticin; GIBCO) 24 h later. Colony formation was determined at 14 to 21 days posttransfection, and cell lines were derived from well-isolated colonies and subcloned.

Genomic cloning and plasmids. pEW53.3 contains the genomic 16-kb p53 gene isolated from the spleen of a normal BALB/c mouse. Molecular cloning of this allele was done as detailed previously (25). Restriction enzyme analysis of several isolated *Eco*RI DNA cloned inserts revealed banding patterns identical to each other and to that of the genomic murine p53 clone CB7 (52). The *Eco*RI insert from one such clone, λHX5-2, was subcloned into the expression vector pECE (16) in both the forward orientation (pEW53.6) and reverse orientation (pEW53.3) with respect to the SV40 early promoter.

Plasmid pLTRp53cG9 encodes a hybrid mouse genomic p53-cDNA construct (14) which expresses a mutant $p53^{Val\text{-}135}$ protein from a Harvey murine sarcoma virus long terminal repeat promoter and was a gift from M. Oren. Plasmid pEC53 encodes a full-length genomic murine p53 gene isolated from the FV-transformed erythroleukemic cell line CB7 (52). This plasmid encodes a mutant $p53^{Pro\text{-}193}$ protein (43). The activated *ras* expression plasmid, pEJ, encodes the genomic T24/EJ *ras* allele from its own promoter (54). pδ2005 encodes the SV40 large T tumor antigen and the neomycin resistance gene and was a gift from M. Bastin (2). Finally, either plasmid pY3 for hygromycin resistance (8) or pSV2neo for geneticin resistance (55) was used as a drug-selectable marker.

Immunoprecipitation and Western immunoblotting. p53 immunoprecipitations were performed as previously described (52) with the following variations. [^{35}S]methionine-labeled cells (3 h) were lysed for 30 min in 1.0 ml of lysis buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris-HCl [pH 8.0], 50 μg of aprotinin [Sigma]) on ice with intermittent shaking. Cellular debris was removed by centrifugation, and the supernatant was precleared by a 40-min incubation in a resuspended pellet of a 10% suspension of crude *Staphylococcus aureus* (250 μl per sample, washed; Sigma). Following recentrifugation, 10^7 trichloroacetic acid-precipitable counts of lysate supernatant was immunoprecipitated for 20 min with the appropriate monoclonal antibody: PAb122, a pan-specific monoclonal antibody against p53 (22); PAb421, a pan-specific monoclonal antibody against p53 (23); PAb246, a murine p53-specific monoclonal antibody sensitive to a conformational epitope on wtp53 protein but generally not reactive with mutant p53 protein species (66); RA3.2C2, a murine p53-specific monoclonal antibody (50); and either PAb419, a monoclonal antibody against SV40 large T antigen (23), or 2 μg of immunoglobulin G2a murine polyclonal antibody (Sigma) as a control. Immune complexes were collected by a subsequent 40-min incubation with 100 μl of a 3% suspension of protein A-Sepharose beads (Pharmacia). Samples were loaded onto a 12.5% polyacrylamide gel in the presence of sodium dodecyl sulfate and electrophoresed at 35 mA. Fixed gels were treated with Enhance (Dupont), dried, and exposed to Eastman Kodak XAR-5 film with Lightning-Plus screens at −70°C.

ras Western immunoblots were performed as outlined previously (59). Blots were then incubated at 4°C overnight with PAb*ras*10 (Dupont), a *ras* pan-specific monoclonal antibody, and visualized by alkaline phosphatase conjugation (Vectastain; Vector).

RESULTS

Mutant p53 rescues from *ras*-induced growth arrest. If the endogenous rat wtp53 is involved in the mechanism of oncogenic *ras*-induced cell cycle arrest, one would predict that loss of wtp53 activity through the introduction of a p53 mutant should relieve the cell of this negative regulation. A second prediction of this model would be that introduction of an exogenous wtp53 allele would not rescue REF52 cells; rather, it would induce a similar growth arrest.

To this end, we have molecularly cloned a full-length genomic murine wtp53 gene from the spleen of a BALB/c *nu*/+ mouse. In Cos cell transient transfection assays, both plasmids express a p53 protein precipitable by the p53-specific monoclonal antibodies PAb122, PAb421, PAbRA 3.2C2, and PAb246 (unpublished results). We then tested these alleles in the classic immortalization and *ras* cotransfection assays of primary rat embryo fibroblasts. While both the $p53^{Val\text{-}135}$- and $p53^{Pro\text{-}193}$-encoding plasmids exhibited these activities, pEW53.3 and pEW53.6 lacked any immortalizing or *ras* cooperating properties (data not shown).

TABLE 1. p53 rescue from *ras*-induced growth arrest

Expt	Transfected plasmids[a]	Transfected gene(s)	Colony formation[b]	Morphology	Stable line[c]
1	pEJ	*ras* alone	≤5	Flat	ND
	pY3	Drug resistance	87	Flat	ND
	pLTRp53cG9	$p53^{Val-135}$ alone	44	Flat	ND
	pLTRp53cG9 + pEJ	$p53^{Val-135}$ + *ras*	21	Refractile	ND
2	pEJ	*ras* alone	≤5	Flat	No
	pδ2005 + pEJ	Large T + *ras*	159	Refractile	Yes
	pY3	Drug resistance	186	Flat	Yes
	pLTRp53cG9 + pEJ	$p53^{Val-135}$ + *ras*	98	Refractile	Yes
3	pEJ	*ras* alone	≤5	Flat	No
	pSV2neo	Drug resistance	225	Flat	Yes
	pLTRp53cG9 + pEJ	$p53^{Val-135}$ + *ras*	200	Refractile	Yes
	pEC53 + pEJ	$p53^{Pro-193}$ + *ras*	35	Refractile	Yes
	pEW53.3	wtp53 alone	≤5	Flat	No
	pEW53.3 + pEJ	wtp53 + *ras*	≤5	Flat	No
	pEW53.6 + pEJ	wtp53 + *ras*	≤5	Flat	No

[a] Transfected plasmids and genes: pEJ, genomic T24/EJ *ras* allele; pδ2005, SV40 large T antigen; pY3, hygromycin resistance gene; pSV2neo, neomycin resistance gene; pLTRp53cG9, $p53^{Val-135}$ (mutant murine p53); pEC53, $p53^{Pro-193}$ (mutant murine p53 full-length genomic clone); pEW53.3, wtp53 (murine p53 full-length genomic clone); pEW53.6, wtp53 (murine p53 full-length genomic clone). All transfections were coprecipitated with the indicated drug-selectable marker. All plasmids used in these transfections were standardized to equimolar equivalents of the p53 coding sequence of pLTRp53cG9 (5 μg of DNA).

[b] Number of drug-selected colonies per transfection.

[c] Ability to establish a stable cell line from a single colony. ND, Not done.

These results are consistent with recently published data for pEW53 or other wtp53-expressing plasmids in negative growth regulation or tumor suppression experiments (13, 18, 26, 43). Considering these results together with the biological activities described in this report, we believe that these plasmids encode a true murine wtp53 under the control of its own promoter (pEW53.3) and subject to normal processing. To control for differences in the wtp53 plasmid constructs, we used plasmid pEC53 (pECE FV-transformed erythroleukemic cell line CB7 genomic p53 gene), which encodes a $p53^{Pro-193}$ conformational mutant that has been extensively characterized (42, 43, 51).

Table 1 shows the results of three independent experiments in which several genes were cotransfected with *ras* into REF52 cells and subsequently assayed for their ability to rescue these cells from *ras*-induced growth arrest. Transfection of SV40 large T antigen plus *ras* results in a colony formation almost equal to the transfection efficiency (as determined by the frequency of drug-selectable colonies in a drug-selectable marker alone transfection). These colonies have a highly transformed phenotype, and cloned cell lines are easily attained. This is in contrast to transfection of the T24/EJ *ras*-encoding plasmid (pEJ) alone into REF52 cells, which consistently results in very low colony formation (<0.5% of the transfection frequency). These colonies rarely go beyond the 20-cell stage and are never able to form stable cloned cell lines. This result is consistent with previously published results showing that SV40 large T antigen is able to rescue REF52 cells normally growth arrested by expression of oncogenic *ras* (20, 27). Transfection of the $p53^{Val-135}$-encoding plasmid pLTRp53cG9 alone does not appear to alter the transfection frequency or the phenotype. This too is consistent with previously published observations for the transfection of other immortalizing nuclear oncogenes alone into the already established REF52 cell line (20, 27, 32).

Both p53 mutant genes were able to rescue REF52 cells from *ras*-induced lethality. The $p53^{Val-135}$-encoding plasmid, pLTRp53cG9, rescued REF52 cells with an efficiency slightly lower than that of the large T antigen. Transfection of pEC53 in the $p53^{Pro-193}$-plus-*ras* assay, however, yielded roughly 15% of the number of colonies of its $p53^{Val-135}$-plus-*ras* counterpart. As transfection conditions were identical (including molar equivalents of each plasmid), this finding likely reflects a difference in stable integration of the much larger genomic allele in the case of pEC53.

The colony-forming ability of wtp53 plus *ras* was no different from the frequency of *ras* alone, indicating that wtp53 is not able to rescue REF52 cells from *ras*-induced growth arrest. The same results were found with transfection of wtp53 alone, which reduced the number of drug-selectable colonies to nearly zero. Only three cell lines were isolated from which nonrefractile cell lines were established. These lines were similar to the parental line in morphology and subsequently shown by Southern analysis not to have incorporated the p53 or *ras* plasmids.

Finally, there are distinct morphological phenotypes of these various cell lines. Figure 1 shows phase-contrast photomicrographs of typical transfectant morphologies. The parental line REF52 is flat, retaining the characteristic fibroblastic shape and very large cytoplasmic content (Fig. 1A). The $p53^{Val-135}$-plus-*ras* transfectants possess a more distinct nucleus and show a dramatic drop in cytoplasmic content (Fig. 1B). Although they retain the star or diamond shape of a fibroblast, they are much smaller and refractile. REF52 cells transfected with $p53^{Pro-193}$ plus *ras* are for the most part similar to the latter cell type but have an interesting subpopulation of highly amorphous cells exhibiting high levels of cell ruffling, scattering, and pseudopodal extensions (Fig. 1C). Under conditions of extensive passaging or variable plating densities, most subclones of these lines retained the expression of this amorphous subpopulation (data not shown). The SV40 large T antigen-plus-*ras* cells were the most refractile, with morphology characteristics of the transformed phenotype (Fig. 1D).

p53 versus *ras* expression. We next determined whether the ability to rescue *ras*-induced growth arrest in REF52 cells is dependent on the level of expression of the cotransfected genes. Immunoprecipitations of extracts from four large T antigen-plus-*ras* [^{35}S]methionine-labeled cell lines are shown in Fig. 2B. Both SV40 large T antigen and p53 coprecipitate

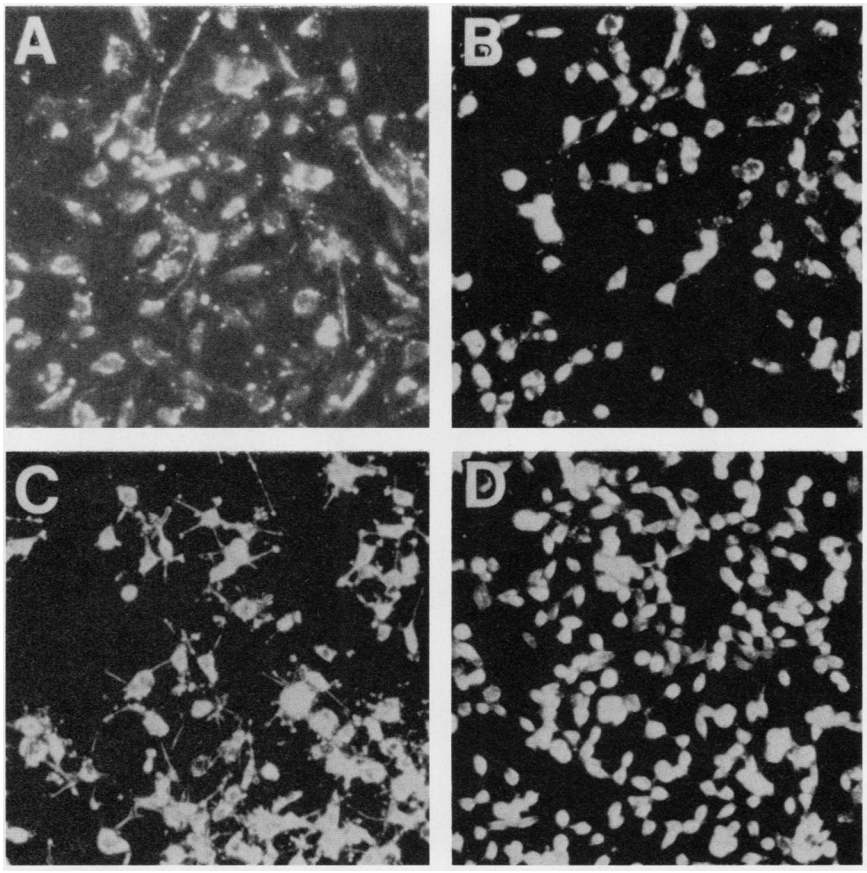

FIG. 1. Phase-contrast photomicrographs of parental REF52 cells (A), $p53^{Val-135}$-plus-*ras* cell line 52LTR/4 (B), $p53^{Pro-193}$-plus-*ras* cell line R53/4 (C), and SV40 large T antigen-plus-*ras* cell line 52δ2005/1 (D). All exposures and fields are the same except for panel A, which was exposed twice as long to visualize the cytoplasm.

in almost equimolar amounts with the p53-specific monoclonal antibody PAb421 (lanes B). Similarly, coprecipitation of both proteins is observed with use of a large T antigen-specific monoclonal antibody, PAb419 (lanes C). The increased stability of p53 in these cells can be explained by their bound state to the SV40 large T antigen. The protein band seen at the apparent molecular weight of p35 is an unrelated immunological cross-reactive protein previously characterized (23).

Expression of p53 in the mutant p53 cotransfections was next examined. All $p53^{Val-135}$-plus-*ras* cell lines derived from individual colonies showed elevated expression of the introduced p53 gene. While there is some variation between cell lines in terms of the relative increases in exogenous p53 expression, all clones show great increases in absolute levels of p53 over the normal low expression of endogenous rat p53 (Fig. 2A, lanes B). Mutant p53 proteins can complex with the cellularly encoded heat shock proteins. This is the presumed identity of the coprecipitating protein seen at apparent molecular sizes of 68 to 72 kDa, as previously described (47). All $p53^{Pro-193}$-plus-*ras*-transfected cell lines showed significantly enhanced p53 protein levels (Fig. 2C). In addition, the $p53^{Pro-193}$ proteins appear to coprecipitate several other cellular proteins with the apparent molecular weights of p70, p60, and p34.

To determine that these observed protein levels were reflective of the cell lines' steady-state p53 expression, we metabolically labeled the cells for a period of 18 h (greater than 50 times the normal endogenous p53 half-life). In cell lines transfected with either p53 mutant, virtually all p53 expressing the pan-reactive PAb421 epitope also expressed the murine-specific RA3.2C2 epitope. Further, the lysates showed little reactivity with the murine wild-type antibody PAb246 (Fig. 3). These results suggest that the majority of the p53 expressed in the $p53^{Val-135}$ or $p53^{Pro-193}$ transfectants is the exogenous mutant p53. We then took the supernatant of the RA3.2C2 immunoprecipitation and subjected it to a second precipitation with RA3.2C2 followed by PAb421 (Fig. 3). The final pan-reactive PAb421 signal was stronger than the preceding murine-specific one, presumably indicating an increased steady-state expression of the endogenous rat p53. This result is consistent with the dominant negative model whereby endogenous p53 protein levels are increased by their oligomerization with the mutant p53.

We next examined the expression of the *ras* protein. Large increases in the expression of activated *ras* protein in the large T antigen-plus-*ras* transfectant lines δ2005/1 and δ2005/3 were detected (Fig. 4). Increased expression of T24/EJ *ras* in REF52 cells is typically accompanied by a reduction or complete loss of the endogenous *ras*. This is visualized by the loss of the normal doublet (processed and unprocessed forms) seen in the parental REF52 cell line and the increased expression of the EJ *ras* running as a band between them.

With the exception of cell line 52LTR/6, all $p53^{Val-135}$-plus-*ras* lines showed increased levels of the EJ *ras* protein.

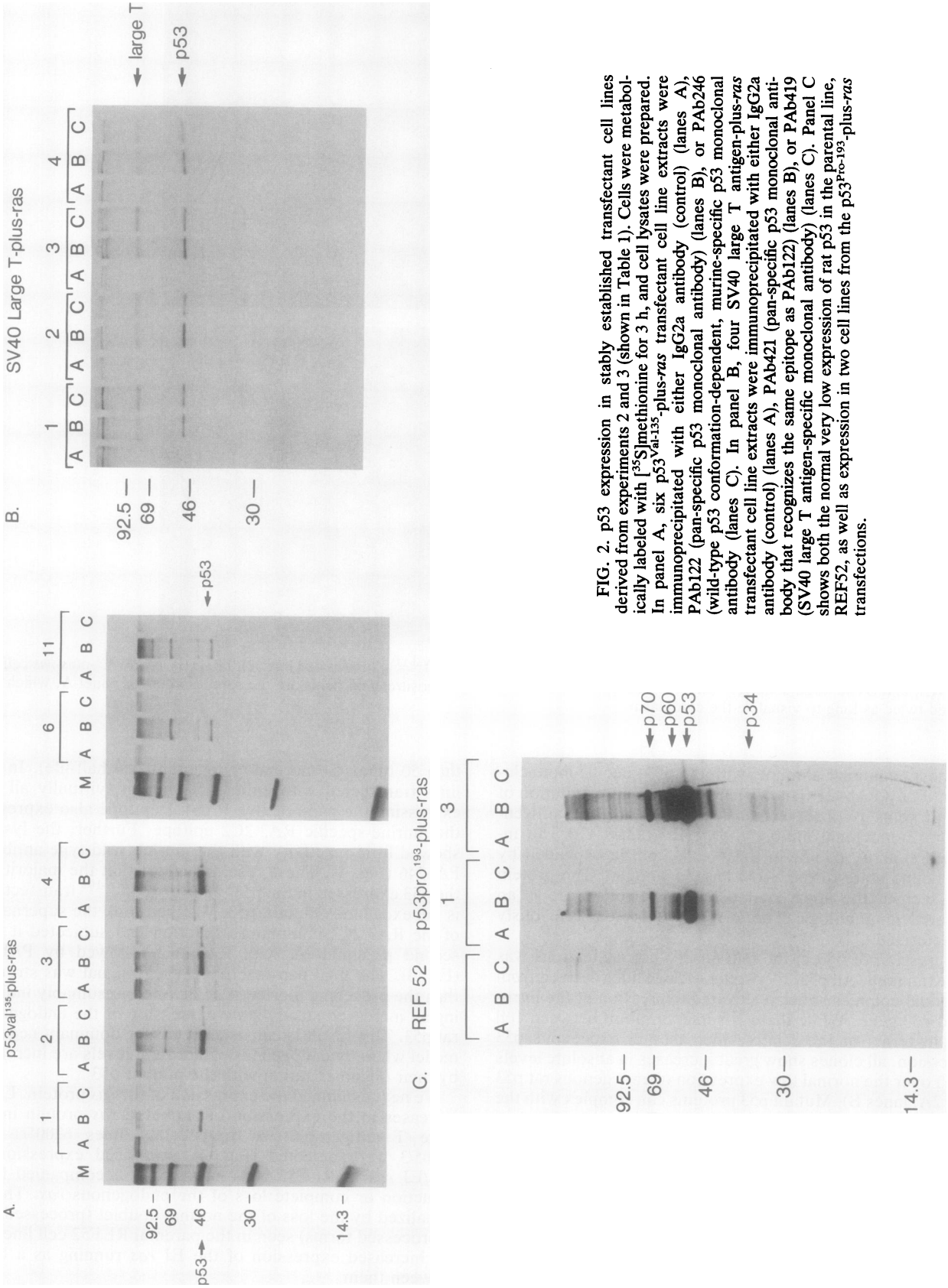

FIG. 2. p53 expression in stably established transfectant cell lines derived from experiments 2 and 3 (shown in Table 1). Cells were metabolically labeled with [^{35}S]methionine for 3 h, and cell lysates were prepared. In panel A, six p53$^{Val-135}$-plus-*ras* transfectant cell line extracts were immunoprecipitated with either IgG2a antibody (control) (lanes A), PAb122 (pan-specific p53 monoclonal antibody) (lanes B), or PAb246 (wild-type p53 conformation-dependent, murine-specific p53 monoclonal antibody (lanes C). In panel B, four SV40 large T antigen-plus-*ras* transfectant cell line extracts were immunoprecipitated with either IgG2a antibody (control) (lanes A), PAb421 (pan-specific p53 monoclonal antibody that recognizes the same epitope as PAb122) (lanes B), or PAb419 (SV40 large T antigen-specific monoclonal antibody) (lanes C). Panel C shows both the normal very low expression of rat p53 in the parental line, REF52, as well as expression in two cell lines from the p53$^{Pro-193}$-plus-*ras* transfections.

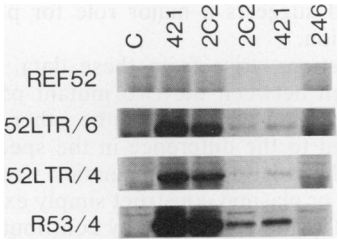

FIG. 3. Steady-state characterization of p53 expression. REF52, $p53^{Val-135}$, $p53^{Val-135}$-plus-*ras*, and $p53^{Pro-193}$-plus-*ras* cell lines (REF52, 52LTR/6, 52LTR/4, and R53/4, respectively) were metabolically labeled with [^{35}S]methionine for 18 h to approximate a steady-state labeling for p53. Prepared extracts were then immunoprecipitated with (left to right) control monoclonal antibody PAb419 (C), p53 pan-reactive monoclonal antibody PAb421 (421), p53 murine-specific monoclonal antibody RA3.2C2 (2C2), supernatant from the previous RA3.2C2 precipitation reprecipitated with monoclonal antibody RA3.2C2 (2C2), supernatant from the second RA3.2C2 precipitation reprecipitated with monoclonal antibody PAb421 (421), and p53 murine wild-type conformation-specific monoclonal antibody PAb246 (246).

Despite the variation in the levels of $p53^{Val-135}$ seen in the transfectants (Fig. 2A), all transfectants express the same level of EJ *ras*. 52LTR/6 was the only cell line to show just endogenous *ras* expression. This line was subsequently determined not to have stably integrated any copies of the EJ *ras* gene. It is interesting to note that this line retained the morphology and characteristics of the parental line.

The $p53^{Pro-193}$-plus-*ras* transfectants (R53/1, -4, -9, and -11), however, show increases in the expression of EJ *ras* equal to or greater than the levels seen in the large T-plus-*ras* lines (Fig. 4). It is important to note that these significant increases in *ras* protein are coincident with the significant increases of $p53^{Pro-193}$ seen over that of the $p53^{Val-135}$ lines.

p53-plus-*ras* complementation to full transformation is allele specific. We next determined whether tumorigenicity was affected by the levels of *ras* expression in the $p53^{Val-135}$, $p53^{Pro-193}$, and large T antigen-plus-*ras* transformant lines. Table 2 shows the tumor frequencies and latency periods of the various transfectants. Only one of nine $p53^{Val-135}$-plus-*ras*-transformed lines had any capacity for tumor formation. 52LTR/11 was very sporadic in its ability to form tumors; the tumors were relatively small and had a very lengthy latency. The $p53^{Pro-193}$-plus-*ras* transfectants, on the other hand, readily formed tumors when injected subcutaneously into nude mice. The tumorigenicity of these cell lines, as measured by the latency period of tumor formation, was slightly slower than that seen with the T antigen-plus-*ras* transfectants.

DISCUSSION

In this study, we provide experimental evidence that the p53 tumor suppressor gene participates in the *ras*-induced negative growth regulation in REF52 cells. Consistent with previous reports, we have shown here that large T antigen-plus-*ras* transformation is very effective in rescuing REF52 cells from *ras*-induced lethality. Immunoprecipitation of either large T antigen or p53 in these transfectants coprecipitates nearly equal amounts of the other. Furthermore, binding of wtp53 protein by large T antigen is coincident with an increased tolerance to the expression of a transfected *ras* gene and a high degree of tumorigenicity.

If the SV40 large T antigen is binding wtp53 to remove its activity as a negative growth regulator, we predicted that introduction of mutant p53 genes in a p53-plus-*ras* transfection of REF52 cells would replace the transforming activity

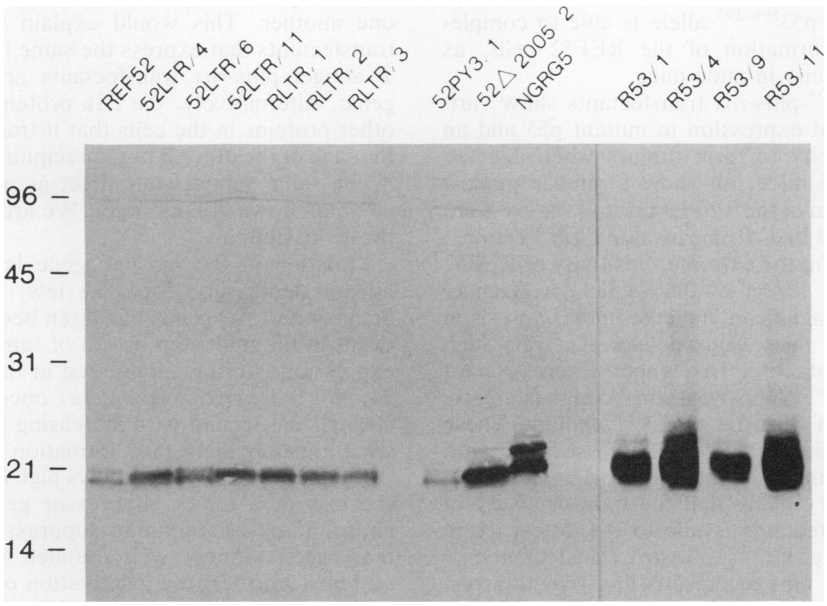

FIG. 4. Western blot analysis of *ras* expression. Cell lines were harvested and adjusted to a final concentration of 10^7 cells per ml. Cell lysates were prepared, and 50-μl samples were subjected to polyacrylamide gel electrophoresis and Western blotting. Blots were incubated with *ras* antibody (PAb*ras*10; Dupont) and visualized by using alkaline phosphatase conjugates. REF52 and 52PY3/1 lanes show normal endogenous *ras* expression of the parental line and transfection control, respectively. $p53^{Val-135}$-plus-*ras* cell lines (52LTR/4, -6, and -11; -RLTR/1, -2, and -3) and $p53^{Pro-193}$-plus-*ras* cell lines (R53/1, -4, -9, and -11) are also shown. 52Δ2005/2 is an SV40 large T antigen-plus-*ras* cell line, and NGRG5 is a positive control line that expresses high levels of v-Ha-*ras*.

TABLE 2. Allele specificity of tumorigenicity[a]

Cell line	Tumor frequency (latency [wk])
pY3/SV2neo	
pY3/1	0/8
ECW/1	0/6
ECW/2	0/6
ECW/3	0/6
T + *ras*	
52δ2005/1	6/6 (1)
52δ2005/2	6/6 (1)
$p53^{Val-135}$ + *ras*	
52LTR/1	0/8
52LTR/2	0/8
52LTR/3	0/8
52LTR/4	0/14
52LTR/6	0/14
52LTR/11	3/14 (10)
RLTR/1	0/6
RLTR/2	0/6
RLTR/3	0/6
$p53^{Pro-193}$ + *ras*	
R53/1	5/6 (3)
R53/4	5/6 (3)
R53/11	6/6 (3)

[a] Tumorigenicity was determined by injection of 5×10^5 cells subcutaneously into BALB/c nude mice. The genes transfected and the names of cloned cell lines expressing them are given. Tumor frequency is the number of tumors formed over the number of injections. All mice were sacrificed at the end of 13 weeks or when tumor growth was judged to be progressive. Latency is the average number of weeks from the original injection to the observation and palpatable confirmation of a subcutaneous tumor at the site of injection.

of large T antigen. We find that introduction of either the $p53^{Val-135}$ or $p53^{Pro-193}$ allele plus *ras* into REF52 cells is able to rescue these cells from *ras*-introduced lethality. These transfectants are further characterized by increased expression of the introduced mutant p53 and oncogenic *ras* genes and by a morphological transformation. Quite unexpectedly, we discovered only the $p53^{Pro-193}$ allele is able to complement *ras* to full transformation of the REF52 cells, as measured by tumorigenicity in nude mice.

Although the $p53^{Val-135}$-plus-*ras* transfectants show various degrees of increased expression in mutant p53 and an extremely limited capacity to form tumors when injected subcutaneously in nude mice, all show a similar modest increase in the expression of the T24/EJ protein. As we were unable to isolate any cell lines from *ras*-alone transfections, our results further confirm the extreme sensitivity of REF52 cells to even very low levels of oncogenic *ras*. Hence, $p53^{Val-135}$ does permit a significant increase in the expression of T24/EJ *ras*. Further, this rescue occurs at a very high percentage of the transfection frequency. Expression of mutant p53 in the $p53^{Pro-193}$-plus-*ras* transfectants is greatly increased over that seen with the $p53^{Val-135}$ mutant. These increases in the expression of $p53^{Pro-193}$ is associated with several p53-coprecipitating proteins and large increases in *ras* expression. It is very likely that the tumorigenicity of $p53^{Pro-193}$-plus-*ras* transfectants is due to the difference in *ras* expression over the $p53^{Val-135}$-plus-*ras* transfectants.

Transfection of wtp53 plus *ras* results in a growth arrest similar to that seen with *ras* alone. It is important to emphasize that the same observations were made in the wtp53-alone transfections. The aberrant expression of wtp53 in REF52 cells is strongly selected against. This is consistent with a wtp53 antiproliferative effect seen in other model systems using either the same wtp53 construct (30) or others (4, 38, 39) and suggests a major role for p53 in negative growth regulation.

Several questions arise from these data. First, are the differences seen between the two mutant p53s a result of increased expression of one over the other, or is it more integrally linked to the difference in the specific mutations themselves? The most direct explanation would be that the $p53^{Pro-193}$ gene or plasmid construct simply expresses higher levels of p53. The increased activity contributed by a highly expressed mutant p53 allows a greater *ras* tolerance in REF52 cells. This may be through a more efficient sequestering of endogenous wild-type p53 or through an increased competition for wtp53 targets or both.

Alternatively, the differences in the p53 mutations could be responsible for the differences in *ras* expression. Recent data from Moshe Oren's laboratory have demonstrated that the $p53^{Val-135}$ mutant encoded by pLTRp53cG9 is temperature sensitive in terms of the stability of its conformational change (39). It is possible there is a significant subpopulation of the $p53^{Val-135}$ proteins which will retain wild-type antiproliferative activity or be stabilized in a wild-type conformation by appropriate substrates. Such a state of equilibrium between mutant $p53^{Val-135}$ and wild-type $p53^{Val-135}$ proteins would explain how the mutant $p53^{Val-135}$ can rescue REF52 cells from *ras*-induced lethality but a residual wild-type $p53^{Val-135}$ activity can hold *ras* expression to levels which are below the threshold of full transformation. The delicate nature of this balance would be further supported by the limited transient capacity of the $p53^{Val-135}$-plus-*ras* cell line 52LTR/11 to form tumors.

Finally, one needs to address the point that the Ad5 E1a protein, which does not bind p53, is as effective as SV40 large T antigen in the rescue and full transformation of REF52 cells by *ras* (20, 53, 61). One explanation is that both E1a and large T antigen bind the retinoblastoma susceptibility gene product RB, another well-characterized antioncogene. It may be that both p53 and RB affect a similar negative growth regulation pathway or work in concert with one another. This would explain why $p53^{Pro-193}$-plus-*ras* transfectants that express the same levels of *ras* as the large T antigen-plus-*ras* transfectants are slightly less tumorigenic. Alternatively, the E1a protein coprecipitates several other proteins in the cells that it transforms (62). It may be that one of the other E1a-coprecipitating proteins is involved in the same suppression effect as p53 but interacts with a common downstream target. We are currently investigating these possibilities.

Mutation in the *ras* oncogene is associated with many human neoplasias (for a review, see reference 5). The activation of *ras* genes has often been shown to be an early event in the multistep model of tumor progression, and its expression is often suppressed in these early stages (9, 17, 34, 46). Increased levels of *ras* oncoprotein expression are strongly associated with increasing levels of tumorigenicity and ultimately metastasis formation (11). It is likely that the subsequent event which allows high *ras* expression would be the loss of a tumor suppressor gene or its activity. The antiproliferative and tumor-suppressing activities of the normal wtp53 is now well founded in experimental model systems. Further, the inactivation of wtp53 by mutation or deletion has been reported in almost every human neoplasia examined and is often a late event in tumor progression (17). We have demonstrated here that the concomitant expression of a dominant-acting mutant p53 gene can overide the normal negative growth regulation in REF52 cells induced by the aberrant expression of an activated *ras*. This rescue is

characterized by a tolerance to very high levels of *ras* expression and full transformation. These data support the model that the normal tumor suppression mechanism of wtp53 is through negative growth regulation induced by aberrant oncogene expression.

ACKNOWLEDGMENTS

We thank H. Earl Ruley for the REF52 cell line, Alain Lavigueur for the concomitant subcloning of pEW53.3 and pEW53.6 from the original viral vectors, and Donald Munroe, Penny Johnson, and Sam Benchimol for sharing their results prior to publication. We also express our gratitude to Mary Sauder, Dave Tough, Bob Choy, Jackie Damen, and Eileen McMillan for their invaluable technical consultations.

G.G.H. is a University of Manitoba Graduate Fellow, S.E.E. is a Research Fellow of the National Cancer Institute of Canada, A.H.G. is a Terry Fox Career Award Scientist of the National Cancer Institute of Canada, and M.M. is a Manitoba Cancer Foundation Career Investigator. This work was supported by grants from the National Cancer Institute of Canada.

REFERENCES

1. **Addison, C., J. R. Jenkins, and H.-W. Sturzbecher.** 1990. The p53 nuclear localisation signal is structurally linked to a $p34^{cdc2}$ kinase motif. Oncogene **5**:423–426.
2. **Asselin, C., and M. Bastin.** 1985. Sequences from polyomavirus and simian virus 40 large T genes capable of immortalizing primary rat embryo fibroblasts. J. Virol. **56**:958–968.
3. **Baker, S. J., E. R. Fearon, J. M. Nigro, S. R. Hamilton, A. C. Preisinger, J. M. Jessup, P. vanTuinen, D. H. Lodbetter, D. F. Barker, Y. Nakamura, R. White, and B. Vogelstein.** 1989. Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. Science **224**:217–221.
4. **Baker, S., S. Markowitz, E. R. Fearon, J. K. V. Willson, and B. Vogelstein.** 1990. Suppression of human colorectal carcinoma cell growth by wild-type p53. Science **249**:912–915.
5. **Barbacid, M.** 1987. *ras* genes. Annu. Rev. Biochem. **56**:779–827.
6. **Ben-David, Y., V. R. Prideaux, V. Chow, S. Benchimol, and A. Berstein.** 1988. Inactivation of the p53 oncogene by internal deletion or retroviral integration in erythroleukemic cells induced by Friend leukemia virus. Oncogene **3**:179–185.
7. **Bishoff, J. R., P. N. Friedman, D. R. Mashak, C. Prives, and D. Beach.** 1990. Human p53 is phosphorylated by p60-cdc2 and cyclin B-cdc2. Proc. Natl. Acad. Sci. USA **87**:4766–4770.
8. **Blochlinger, K., and H. Diggelmann.** 1984. Hygromycin B phosphotransferase as a selectable marker for DNA transfer experiments with higher eucaryotes. Mol. Cell. Biol. **4**:2929–2931.
9. **Bremner, R., and A. Balmain.** 1990. Genetic changes in skin tumor progression: correlation between presence of a mutant *ras* gene and loss of heterozygosity on mouse chromosome 7. Cell **61**:407–417.
10. **Clarke, C. F., K. Cheng, A. B. Frey, R. Stein, P. W. Hinds, and A. J. Levine.** 1988. Purification of complexes of nuclear oncogene p53 with rat and *Escherichia coli* heat shock proteins: in vitro dissociation of *hsc70* and *dnaK* from murine p53 by ATP. Mol. Cell. Biol. **8**:1206–1215.
11. **Egan, S. E., G. A. McClarty, L. Jarolim, J. A. Wright, I. Spiro, G. Hager, and A. H. Greenberg.** 1987. Expression of H-*ras* correlates with metastatic potential: evidence for direct regulation of the metastatic phenotype in 10T1/2 and NIH 3T3 cells. Mol. Cell. Biol. **7**:830–837.
12. **Eliyahu, D., N. Goldfinger, O. Pinhasi-Kimhi, G. Shaulsky, Y. Shurnik, N. Arai, V. Rotter, and M. Oren.** 1988. Meth A fibrosarcoma cells express two transforming mutant p53 species. Oncogene **3**:313–321.
13. **Eliyahu, D., D. Michalovitz, S. Eliyahu, O. Pinkhasi-Kimhi, and M. Oren.** 1989. Wild-type p53 can inhibit oncogene-mediated focus formation. Proc. Natl. Acad. Sci. USA **86**:8763–8767.
14. **Eliyahu, D., D. Michalovitz, and M. Oren.** 1985. Overproduction of p53 antigen makes established cells highly tumorigenic. Nature (London) **316**:158–160.
15. **Eliyahu, D., A. Raz, P. Gruss, D. Givol, and M. Oren.** 1984. Participation of p53 cellular tumour antigen in transformation of normal embryonic cells. Nature (London) **312**:646–649.
16. **Ellis, L., E. Clauser, D. O. Morgan, M. Edery, R. A. Roth, and W. J. Rutter.** 1986. Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin stimulated kinase activity and uptake of 2-deoxyglucose. Cell **45**:721–732.
17. **Fearon, E. R., and B. Vogelstein.** 1990. A genetic model for colorectal tumorigenesis. Cell **61**:759–767.
18. **Finlay, C. A., P. W. Hinds, and A. J. Levine.** 1989. The p53 proto-oncogene can act as a suppressor of transformation. Cell **57**:1083–1093.
19. **Finlay, C. A., P. W. Hinds, T.-H. Tan, D. Eliyahu, M. Oren, and A. J. Levine.** 1988. Activating mutations for transformation by p53 produce a gene product that forms an hsc-p53 complex with an altered half life. Mol. Cell. Biol. **8**:531–539.
20. **Franza, B. R., Jr., K. Maruyama, J. I. Garrels, and H. E. Ruley.** 1986. In vitro establishment is not a sufficient prerequisite for transformation by activated ras oncogenes. Cell **44**:409–418.
21. **Gannon, J. V., R. Greaves, R. Iggo, and D. P. Lane.** 1990. Activating mutations in p53 produce a common conformational effect. A monoclonal antibody specific for the mutant form. EMBO J. **9**:1595–1602.
22. **Gurney, E. G., R. D. Harrison, and J. Fenno.** 1980. Monoclonal antibodies against simian virus 40 large T antigens: evidence for distinct subclasses of large T antigen and for similarities among nonviral T antigens. J. Virol. **34**:752–763.
23. **Harlow, E., L. V. Crawford, D. C. Pim, and N. M. Williamson.** 1981. Monoclonal antibodies specific for simian virus 40 tumor antigens. J. Virol. **39**:861–869.
24. **Herskowitz, I.** 1987. Functional inactivation of genes by dominant negative mutations. Nature (London) **329**:219–222.
25. **Hick, G. G., and M. Mowat.** 1988. Integration of Friend murine leukemia virus into both alleles of the p53 oncogene in an erythroleukemic cell line. J. Virol. **62**:4752–4755.
26. **Hinds, P., C. Finlay, and A. J. Levine.** 1989. Mutation is required to activate the p53 gene for cooperation with the *ras* oncogene and transformation. J. Virol. **63**:739–746.
27. **Hirakawa, T., and H. E. Ruley.** 1988. Rescue of cells from *ras* oncogene-induced growth arrest by a second, complementing, oncogene. Proc. Natl. Acad. Sci. USA **85**:1519–1523.
28. **Jenkins, J. R., K. Rudge, and G. A. Currie.** 1984. Cellular immortalization by a cDNA clone encoding the transformation-association phosphoprotein p53. Nature (London) **312**:651–654.
29. **Jenkins, J. R., and H.-W. Sturzbecher.** 1988. The p53 oncogene, p. 403–423. *In* E. P. Reddy, A. M. Skalka, and T. Curran (ed.), The oncogene handbook. Elsevier Science Publications, Amsterdam.
30. **Johnson, P., D. Gray, M. Mowat, and S. Benchimol.** 1991. Expression of wild-type p53 is not compatible with continued growth of p53-negative tumor cells. Mol. Cell. Biol. **11**:1–11.
31. **Kelekar, A., and M. D. Cole.** 1986. Tumorigenicity of fibroblast lines expressing the adenovirus E1a, cellular p53, or normal c-*myc* genes. Mol. Cell. Biol. **6**:7–14.
32. **Kohl, N. E., and H. E. Ruley.** 1987. Role of c-*myc* in the transformation of REF52 cells by viral and cellular oncogenes. Oncogene **2**:41–48.
33. **Kraiss, S., A. Quaiser, M. Oren, and M. Montenarh.** 1988. Oligomerization of oncoprotein p53. J. Virol. **62**:4737–4744.
34. **Kumar, R., S. Sukumar, and M. Barbacid.** 1990. Activation of *ras* oncogenes preceding the onset of neoplasia. Science **248**:1101–1104.
35. **Lane, D. P., and S. Benchimol.** 1990. p53: oncogene or anti-oncogene? Genes Dev. **4**:1–8.
36. **Levine, A. J., and J. Momand.** 1990. Tumor suppressor genes: the p53 and retinoblastoma sensitivity genes and gene products. Biochim. Biophys. Acta **1032**:119–136.
37. **Masuda, H., C. Miller, H. P. Koeffler, H. Battifora, and M. J. Kline.** 1987. Rearrangements of the p53 gene in human osteogenic sarcomas. Proc. Natl. Acad. Sci. USA **84**:7716–7719.
38. **Mercer, W. E., M. Amin, G. J. Sauve, E. Appella, S. J. Ullrich, and J. W. Romano.** 1990. Wild type human p53 is antiprolifer-

ative in SV40-transformed hamster cells. Oncogene **5**:973–980.
39. **Michalovitz, D., O. Halevey, and M. Oren.** 1990. Conditional inhibition of transformation and of cell proliferation by a temperature-sensitive mutant of p53. Cell **62**:671–680.
40. **Milner, J., and A. Cook.** 1986. The cellular tumour antigen p53: Evidence for transformation-related immunological variants of p53. Virology **154**:21–30.
41. **Milner, J., and S. Milner.** 1981. SV40-53K antigen: a possible role for 53K in normal cells. Virology **112**:785–788.
42. **Mowat, M., A. Cheng, N. Kimura, A. Bernstein, and S. Benchimol.** 1985. Rearrangements of the cellular p53 gene in erythroleukemic cells transformed by Friend virus. Nature (London) **314**:633–636.
43. **Munroe, D. G., J. W. Peacock, and S. Benchimol.** 1990. Inactivation of the cellular p53 gene is a common feature of Friend virus-induced erythroleukemia: relationship of inactivation to dominant transforming alleles. Mol. Cell. Biol. **10**:3307–3313.
44. **Nigro, J. M., S. J. Baker, A. C. Preisinger, J. M. Jessup, R. Hostetter, K. Cleary, S. H. Bigner, N. Davidson, S. Baylin, P. Devilee, T. Glover, F. S. Collins, A. Weston, R. Modali, C. C. Harris, and B. Vogelstein.** 1989. Mutations in p53 gene occur in diverse human tumor types. Nature (London) **342**:705–708.
45. **Parada, L. F., H. Land, R. A. Weinberg, D. Wolf, and V. Rotter.** 1984. Cooperation between genes encoding p53 tumour antigen and ras in cellular transformation. Nature (London) **312**:649–651.
46. **Paterson, H., B. Reeves, R. Brown, A. Hall, M. Furth, J. Bos, P. Jones, and C. Marshall.** 1987. Activated N-*ras* controls the transformed phenotype of HT1080 human fibrosarcoma cells. Cell **51**:803–812.
47. **Pinhasi-Kimhi, O., D. Michalovitz, A. Ben-Zeev, and M. Oren.** 1986. Specific interaction between the p53 cellular tumour antigen and major heat shock proteins. Nature (London) **320**: 182–185.
48. **Reich, N. C., and A. J. Levine.** 1984. Growth regulation of a cellular tumour antigen, p53, in nontransformed cells. Nature (London) **308**:199–201.
49. **Ridley, A. J., H. F. Paterson, M. Noble, and H. Land.** 1988. *ras*-mediated cell cycle arrest is altered by nuclear oncogenes to induce Schwann cell transformation. EMBO J. **7**:1635–1645.
50. **Rotter, V., M. A. Boss, and D. Baltimore.** 1981. Increased concentration of an apparently identical cellular protein in cells transformed by either Abelson murine leukemia virus or other transforming agents. J. Virol. **38**:336–346.
51. **Rovinski, B., and S. Benchimol.** 1988. Immortalization of rat embryo fibroblasts by the cellular p53 oncogene. Oncogene **2**:445–452.
52. **Rovinski, B., D. Munroe, J. Peacock, M. Mowat, A. Bernstein, and S. Benchimol.** 1987. Deletion of 5′-coding sequences of the cellular p53 gene in mouse erythroleukemia: a novel mechanism of oncogene regulation. Mol. Cell. Biol. **7**:847–853.
53. **Ruley, H. E.** 1983. Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature (London) **304**:602–606.
54. **Shih, C., and R. A. Weinberg.** 1982. Isolation of a transforming sequence from a human bladder carcinoma cell line. Cell **29**:161–169.
55. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1**:327–341.
56. **Sturzbecher, H.-W., P. Chumakov, W. J. Welch, and J. R. Jenkins.** 1987. Mutant p53 proteins bind p53 hsp72/73 cellular heat-shock-related proteins in SV40-transformed monkey cells. Oncogene **1**:201–211.
57. **Sturzbecher, H.-W., T. Maimets, P. Chumakov, R. Brain, C. Addison, V. Simanis, K. Rudge, R. Philip, M. Grimaldi, W. Court, and J. R. Jenkins.** 1990. p53 interacts with $p34^{cdc2}$ in mammalian cells: implications for cell cycle control and oncogenesis. Oncogene **5**:795–801.
58. **Takahashi, T., M. M. Nau, I. Chiba, M. J. Birrer, R. K. Rosenberg, M. Vinocour, M. Levitt, H. Pass, A. D. Gazdar, and J. D. Minna.** 1990. p53: a frequent target for genetic abnormalities in lung cancer. Science **246**:491–494.
59. **Tough, D., and D. A. Chow.** Nat. Immun. Cell Growth Regul., in press.
60. **Tuck, S. P., and L. V. Crawford.** 1989. Overexpression of normal human p53 in established fibroblasts leads to their tumorigenic conversion. Oncogene Res. **4**:81–89.
61. **Velich, A., and E. Ziff.** 1988. Adenovirus E1A *ras* cooperation activity is separate from its positive and negative transcription regulatory function. Mol. Cell. Biol. **8**:2177–2183.
62. **Whyte, P., N. M. Williamson, and E. Harlow.** 1989. Cellular targets for transformation by the adenovirus E1A proteins. Cell **56**:67–75.
63. **Wolf, D., S. Admon, M. Oren, and V. Rotter.** 1984. Abelson murine leukemia virus-transformed cells that lack p53 protein synthesis express aberrant p53 mRNA species. Mol. Cell. Biol. **4**:552–558.
64. **Wolf, D., S. Admon, M. Oren, and V. Rotter.** 1984. Major deletions in the gene encoding the p53 tumor antigen cause lack of p53 expression in HL-60 cells. Proc. Natl. Acad. Sci. USA **82**:790–794.
65. **Wolf, D., N. Harris, and V. Rotter.** 1984. Reconstitution of p53 expression in a nonproducer Ab-MuLV-transformed cell line by transfection of a functional p53 gene. Cell **38**:119–126.
66. **Yewdell, J. W., J. V. Gannon, and D. P. Lane.** 1986. Monoclonal antibody analysis of p53 expression in normal and transformed cells. J. Virol. **59**:444–452.